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3. Janicke et al, Sem. Throm. Hemostasis, 1991, 17:303-312)

4. Nekarda et al (Cancer Res., 1994, 54:2900-2907)

5. Grandahl-Hansen et al, 1993, Cancer Research 53:1513-1521)

6. JOURNAL OF NEURO-ONCOLOGY, (1994) Vol. 22, No. 2, pp. 139-151.

7. INTERNATIONAL JOURNAL OF ONCOLOGY, (MAR 1994) Vol. 4, No. 3, pp. 717-721.

8. Biol.Chem.Hoppe Seyler (376, No. 5, 259-67, 1995) 2 Fig. 67 Ref.

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GASTROENTEROLOGY 1994;107:1449–1456

Inactive Urokinase and Increased Levels of Its Inhibitor Type 1 in Colorectal Cancer Liver Metastasis

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See editorial on page 1555.

Background/Aims: **Human colorectal carcinogenesis was previously found to be associated with an increased urokinase-type plasminogen activator expression, both in antigen and activity, accompanied by simultaneously enhanced levels of plasminogen activator inhibitors type 1 and type 2. This increased proteolytic activity may contribute to invasive growth and metastasis of the tumors.** ***Methods:*** **In the present study, homogenates of liver metastases, primary colorectal carcinomas, and adjacent normal tissues were evaluated regarding the level and composition of urokinase, tissue-type plasminogen activator, and plasminogen activator inhibitors.** ***Results:*** **Concentrations of urokinase were significantly increased in primary carcinomas and liver metastases compared with normal tissues, whereas tissue-type plasminogen activator levels were significantly decreased. Liver metastases showed, in contrast to the carcinomas, hardly any activity of plasminogen activators, which could be attributed to the enhanced presence of the inactive proenzyme form of urokinase in combination with more complexes of plasminogen activators with inhibitors. Furthermore, liver metastases had an eightfold higher content of inhibitor type 1 compared with the primary carcinomas. The excess of inhibitors was confirmed by addition of plasminogen activators to metastasis homogenates, which resulted in increased complex formation.** ***Conclusions:*** **Colorectal cancer metastasis in the liver is associated with an inactivation of the enhanced urokinase cascade, which might allow tumor cells to settle in the liver.**

The process of tumor cell invasion and metastasis involves sequential breakdown and reestablishment of the extracellular matrix. Plasminogen activation seems to be an important mechanism in the degradation of a broad spectrum of substrates in this matrix. Conversion of plasminogen into active plasmin is regulated by a complex system containing activators, receptors, and activator inhibitors.[1–3] Extracts of carcinomas of different origin contain increased levels of mainly the urokinase type of plasminogen activator (u-PA) compared with normal tissue extracts.[4–6] Immunohistological studies have indeed shown intensive staining with antibodies against u-PA in neoplastic tissues of the stomach, colon, breast, and lung, particularly at sites of invasive growth.[4,6–16] Other experiments indicate that u-PA plays a key role in tumor proliferation and metastasis. Antibodies against u-PA have been found to prevent extracellular matrix degradation by Rous sarcoma virus–transformed chick fibroblasts[17] and human melanoma cell lines.[18] Ossowski and Reich[19] have shown inhibition of metastasis, but not of primary growth, of human squamous carcinoma cells in chick embryos by antibodies to u-PA. In vitro and in vivo studies with human tumor cell lines from melanomas, breast, lung, stomach, and colon show strong correlations of u-PA levels of the cells with invasive and metastatic potential.[12–24]

Markus et al.[12] and Harvey et al.[13] have compared the secretion rate of u-PA in short-term organ cultures of primary and metastatic colon tumors. The secreted activities of plasminogen activators of liver metastases were lower than those of the primary colon tumors, which is in agreement with the microthrombus theory of the carcinoma-metastasis cascade. However, extracts of bone metastasis from prostatic carcinomas have been found to contain a significantly higher amount of u-PA activity than the primary tumors.[5] Recent u-PA localization studies in adenocarcinomas of the breast, colon, and lung and their corresponding lymph node metastases in general showed more u-PA–positive tumor cells in the metastatic lesion than in the original carcinoma.[6,25] In a preliminary study, Jänicke et al.[7] found the u-PA content

Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; PAI, plasminogen activator inhibitor; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

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in extracts of tumor-occupied lymph nodes not to be different from primary breast carcinomas of the same patients, whereas the concentration of plasminogen activator inhibitor (PAI) 1 was twice as high in the metastases. These apparently controversial results show the complexity of the matter. The different nature of the primary tumors, the existence of two different activators and of an inactive proenzyme form of u-PA, the influence of the specific inhibitors PAI-1 and PAI-2, and the presence of receptors on the tumor cells complicate the study on the role of plasminogen activators in invasion and metastasis.[26]

In this study, we have evaluated the presence of plasminogen activators u-PA and tissue-type plasminogen activator (t-PA), plasminogen activator inhibitors PAI-1 and PAI-2, and complexes between activators and inhibitors in homogenates of human colorectal carcinomas and liver metastases. The neoplastic tissues were compared with the corresponding normal tissues and with tissues of liver affected by other diseases. The results might contribute to a better understanding of the role of plasminogen activation in the process of tumor invasion and metastasis.

Materials and Methods

Patients

Twenty-five patients underwent a partial liver resection, providing us with 14 representative parts of colorectal metastasis: 3 from livers with focal nodular hyperplasia, 3 from hepatocellular carcinomas, and 5 from miscellaneous liver diseases. Representative parts of adjacent macroscopically normal tissue were selected if possible. We also had frozen tissue of the former resected primary colorectal carcinoma and normal mucosa from four of the patients. Tumor tissue and adjacent normal mucosa of eight patients operated on for colorectal carcinoma were used as extra reference tissue. All tissues were immediately frozen at −70°C until analysis; adjacent fragments of all samples were histologically evaluated by pathological examination to confirm the diagnosis of tissue origin and disease.

Tissue Extraction and Protein Concentration

Tissue specimens were homogenized in 1 mL 0.1% (vol/vol) Tween 80 and 0.1 mol/L Tris-HCl (pH 7.5) per 60 mg wet tissue as previously described.[27] The homogenates were centrifuged twice at $8 \times 10^3 g$ for 2.5 minutes at 4°C. Protein concentration of the supernatants was determined by the method of Lowry et al.[28]

Plasminogen Activator Activity Assay

Activities of u-PA and t-PA were measured by a spectrophotometric enzyme assay as previously described.[29] In brief, tissue extract was incubated with plasminogen, fragments of fibrinogen, and the chromogenic plasmin substrate S-2251 (Kabi, Stockholm, Sweden) to detect total plasminogen activator activity. t-PA and u-PA activities were determined by adding specific inhibiting antibodies against t-PA and u-PA, rabbit anti-human t-PA immunoglobulin G, and goat anti-human u-PA immunoglobulin M/immunoglobulin D, respectively, to parallel incubations and calculating the amount of inhibition. u-PA and t-PA standard preparations (batch no. 66/46 and 83/517, respectively; National Institute of Biological Standards and Control, London, England) were included. The inhibiting antibodies used were monospecific, showed no cross-reactivity, and blocked maximum standard u-PA and t-PA completely.

Enzyme-Linked Immunosorbent Assay for u-PA

The sandwich enzyme-linked immunosorbent assay (ELISA) for u-PA was performed according to Binnema et al.[30] Rabbit anti–u-PA was used as catching antibody and after incubation of the samples; affinopurified goat anti-u-PA immunoglobulin G (0.8 μg/mL) was added and incubated. After washing, 100 μL of an optimal dilution of donkey anti-goat immunoglobulin G conjugated with alkaline phosphatase was added, and 100 μL *p*-nitrophenyl-phosphate (1 mg/mL) was used as substrate. The amount of u-PA antigen in the samples was calculated from a nine-point standard curve of u-PA (0–3.3 ng/mL).

ELISA for t-PA

t-PA antigen was measured essentially as described by Rijken et al.[31] Goat anti–t-PA was used as catching antibody, an anti–t-PA horseradish peroxidase conjugate (Biopool, Umeå, Sweden) was used as second antibody, and 3,3′,5,5′tetramethylbenzidine was used as substrate. Absolute quantities of t-PA antigen in the samples were calculated from an eight-point standard curve of t-PA (Biopool; 0–4 ng/mL).

ELISA for PAI-1

Total PAI-1 antigen (i.e., latent, active, and complexed PAI-1) was determined using the Tintelize PAI-1 ELISA (Biopool) without prior denaturation of the samples as previously described.[32] In brief, mouse monoclonal anti-human PAI-1 was used as catching antibody. After incubation with the tissue homogenates, a goat polyclonal anti-human PAI-1, conjugated to peroxidase, was used to form a "sandwich" ELISA; orthophenylenediamine was added as substrate. The assay included the use of quenching and nonspecific antibodies to exclude falsely elevated results. To increase the sensitivity of the assay sample, volumes of up to 40 μL were used instead of the recommended 20 μL, resulting in a detection limit of 0.3 ng/mL.

ELISA for PAI-2

The determination of PAI-2 antigen was performed using the Tintelize PAI-2 ELISA (Biopool) as previously re-

ported.[32] The first antibody used was mouse monoclonal anti-human PAI-2, and the second was goat polyclonal anti–PAI-2 immunoglobulin G conjugated to peroxidase. Orthophenylenediamine was added as substrate. Unspecific response was excluded using quenching antibodies. The detection limit was decreased to 0.5 ng/mL by using 50 μL homogenate instead of 20 μL and by increasing sample incubation, conjugate incubation, and substrate incubation times.

Zymography

Tissue extracts were incubated for 1 hour (2% [wt/vol] sodium dodecyl sulfate at 37°C) to induce activator activity in PA-PAI complexes.[33] Electrophoresis of the samples took place on 10% polyacrylamide gels with sodium dodecyl sulphate. Plasminogen activator activities were visualized on agarose underlay gels containing plasminogen and fibrin.[34]

Bioimmunoassay for u-PA

This assay is a combination of the u-PA ELISA and the enzymatic determination of u-PA in the chromogenic assay and detects u-PA and pro-u-PA separately.[35] Total u-PA antigen was immunoimmobilized as in the ELISA. After incubation of the samples, four wells of the microtiter plate were incubated with plasmin (30 minutes; 14 U/mL) to convert pro-u-PA to active u-PA and four wells with buffer (0.01% [vol/vol] Tween 80, 0.02% [vol/vol] NaN_3, 0.1% [wt/vol] bovine serum albumin in phosphate-buffered saline). After extensive washing, the u-PA activities were spectrophotometrically measured using plasminogen and S-2251 as substrate. Enzyme amounts were calculated from a standard curve of u-PA (batch 66/46; National Institute of Biological Standards and Control, London, England). Specificity was checked by inclusion of activity-inhibiting goat anti-human u-PA immunoglobulin M/immunoglobulin D in four of the wells. Pro-u-PA was calculated by subtraction of plasmin-activated total u-PA activity and the nonactivated u-PA activity.

Statistical Analysis

Results are given as mean ± SEM. Differences between group means were tested for significance using Student's t test with separate variance estimate if the SDs were significantly different according to the F test. Differences were considered significant when $P < 0.05$.

Results

The mean amounts of u-PA in homogenates of colorectal carcinomas and liver metastases compared with normal colorectal mucosa and normal liver tissue are shown in Figure 1. The concentration of u-PA antigen in colorectal carcinomas and liver metastases was significantly increased compared with their respective control tissues. This high antigen level was accompanied by a high u-PA activity in the primary carcinomas but, in contrast, liver metastases hardly showed any u-PA activity (Figure 1*A*). The concentration of t-PA antigen was significantly lower in neoplastic tissues compared with their respective normal tissues, which was associated with a decrease or absence of t-PA activity in the carcinomas and metastases, respectively (Figure 1*B*).

Zymography of the homogenates from neoplastic and normal control tissues showed good agreement with the results of the enzymatic activity assay, i.e., increased u-PA activity and decreased t-PA activity in colon carcinomas compared with normal colon tissue and virtually no t-PA or u-PA activity in liver metastases or normal liver tissue (Figure 2*A*). Especially colon carcinomas and metastases and, to some extent, normal livers showed plasminogen activator activity in high-molecular-weight regions (95–110 kilodaltons) of the zymogram, representing complexes of either t-PA or u-PA with plasminogen activator inhibitors (Table 1). After prolonged exposure of the underlay to the gel, eventually weak u-PA lysis bands appeared in most of the metastasis homogenates, suggesting the presence of u-PA in the proenzyme form. The portion of u-PA that was present in the active form or plasmin-activatable proenzyme form was determined with a bioimmunoassay for u-PA (Table 2). The percentage of pro-u-PA in the colorectal carcinomas (n = 12) was found to be 71% ± 7% and even 97% ± 2% in the metastases (n = 14).

Regarding the inhibitors, we found the mean antigen concentration of PAI-1 in liver metastases to be significantly higher than in all of the other tissues, even compared with the primary colorectal carcinomas. Less pronounced differences were found with PAI-2. Normal liver tissue contained the lowest level of PAI-2 antigen, whereas colon carcinomas showed the highest concentration (Figure 3). These differences were found to be not significant. At least part of the PAI antigen in the homogenates of metastases seemed to be present in an active form because addition of external t-PA or u-PA to the extracts resulted in increased lysis bands on zymograms in (high molecular weight) complex regions at the cost of free uncomplexed regular t-PA and u-PA lysis zones (Figure 2*B*).

The concentrations of u-PA and the inhibitors found in colorectal cancer–related liver tissues were also compared with those in normal liver, liver cell carcinomas, and liver focal nodular hyperplasia (Table 3). There were no significant differences in u-PA or inhibitor concentrations between normal liver tissue adjacent to colorectal cancer metastases resections and normal liver tissue obtained from resections because of other diseases. Noteworthy was the high concentration of PAI-1 in liver cell carcinomas, which was comparable to the level in liver

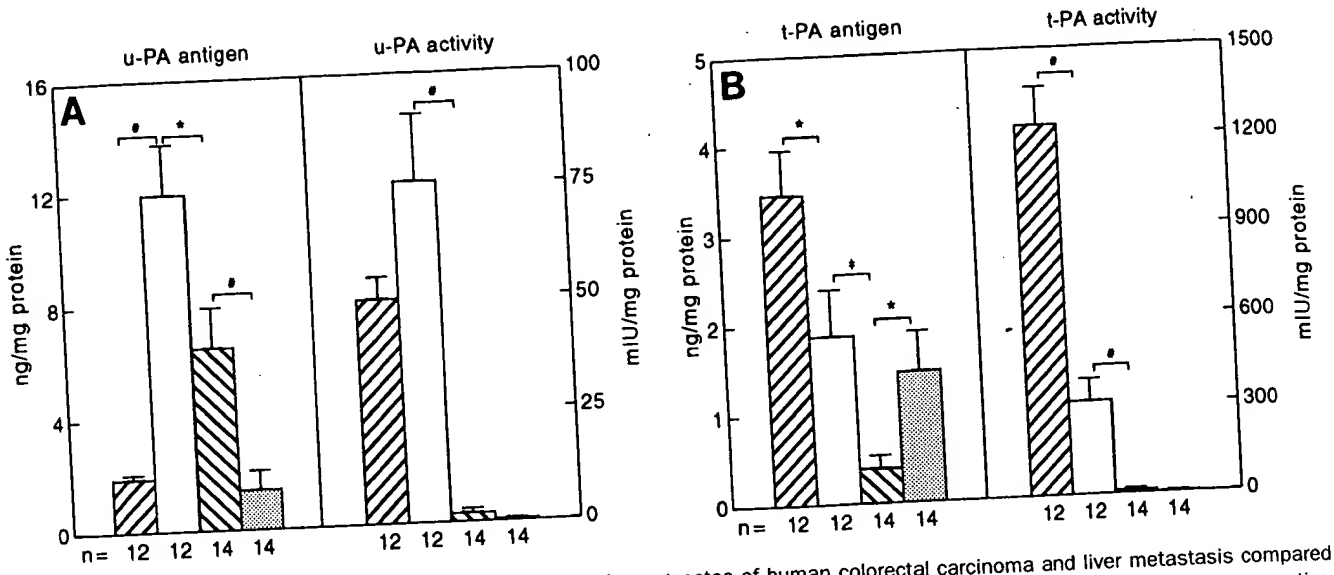

Figure 1. Concentration and activity of (*A*) u-PA and (*B*) t-PA in homogenates of human colorectal carcinoma and liver metastasis compared with adjacent normal tissues (mean ± SEM). Antigen levels were determined with specific ELISAs and activities of both plasminogen activators with a chromogenic assay. Significance of differences: $*P < 0.05$; $^{\dagger}P < 0.02$, $^{\#}P < 0.005$. ▨, normal colon; □, carcinoma; ▧, metastasis; ▩, normal liver.

metastases of colorectal cancer, with the absence of a concomitant increase in u-PA in this tissue type.

Discussion

The association between elevated levels of plasminogen activators, particularly u-PA, and neoplastic growth has been shown in various studies. We previously showed that colorectal neoplasia is associated with an increase in u-PA antigen and u-PA activity[36] and that the latter is not inhibited by enhanced PAI-1 and PAI-2 concentrations.[32] The most interesting finding in the present study is the total absence of plasminogen activator activity in homogenates of liver metastases of colorectal carcinomas, despite the presence of an increased u-PA antigen concentration. This inactivity of the antigen is partly explained by the large percentage of the inactive proform of u-PA. More importantly, however, zymographic analysis showed that a considerable amount of the urokinase antigen seemed to be inactivated by complex formation with specific inhibitors. Increased antigen con-

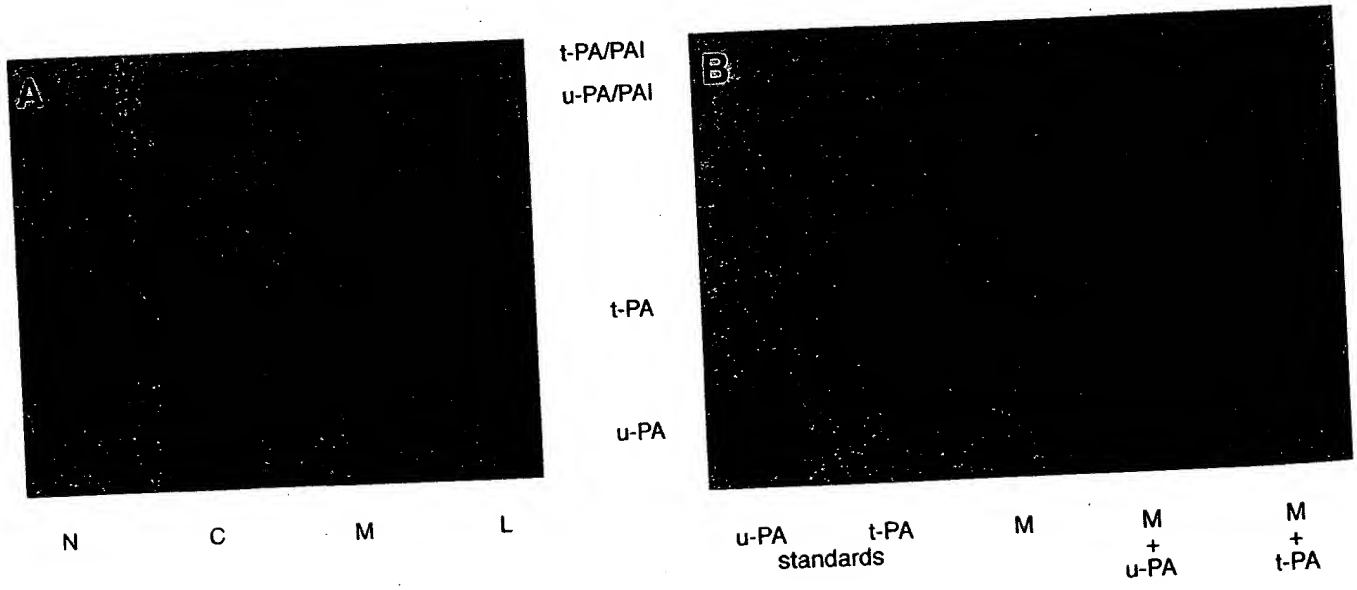

Figure 2. (*A*) Zymographic analysis of plasminogen activator activity in homogenates of human colorectal and liver tissues from one patient. (*B*) Zymographic analysis of liver metastasis tissue of the same patient with or without exogenous u-PA or t-PA added to the homogenate.

Table 1. Number of Homogenates in Which Specific Lysis Bands Appeared After Zymography of Tissue Homogenates of Normal Colon, Colorectal Carcinoma, Liver Metastasis, and Normal Liver

Tissue	n	u-PA lysis bands	t-PA lysis bands	t-PA complex	u-PA complex
Normal colon	11	5	11	3[b]	1[b]
Carcinoma colon	12	12	8	10	5
Liver metastasis	14	0/13[a]	0[b]	13	9
Normal liver	14	0/4[a]	1[b]	10	1[b]

NOTE. Homogenates were incubated with sodium dodecyl sulfate and electrophoresed on sodium dodecyl sulfate–polyacrylamide gels. Plasminogen activator activity lysis bands were scored on underlay gels containing plasminogen and fibrin.
[a]Lysis appeared only after prolonged exposure.
[b]Very poor lysis.

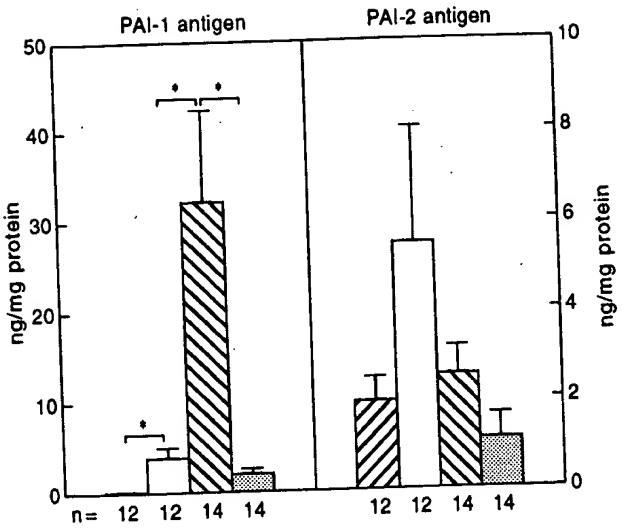

Figure 3. Concentration of plasminogen activator inhibitors PAI-1 and PAI-2 in homogenates of normal colon, colorectal carcinoma, liver metastasis, and normal liver (mean ± SEM). Antigen levels were determined with specific ELISAs. Significance of differences: $*P < 0.02$. ▨, normal colon; □, carcinoma; ▧, metastasis; ▩, normal liver.

centrations of PAI-1 in homogenates of metastases support this observation. Exogenous plasminogen activators added to these homogenates were able to form complexes visualized by zymography, indicating that an excess of inhibitors was present in the uncomplexed active form. It has been shown that PAI-1 can prevent the conversion of pro–u-PA into the active form in plasma[37] and that PAI-1 regulates the internalization and degradation of u-PA by tumor cells in vitro.[38] The presence of extremely high concentrations of PAI-1 in liver metastasis homogenates results in the absence of u-PA activity not only by forming complexes with already activated u-PA, as found in this study, but probably also by preventing the processing of pro–u-PA to its active form. Because of the known spatially controlled action of u-PA,[39,40] we cannot conclude that no activator activity in liver metastasis is present in vivo. In primary stomach and colorectal carcinomas, the central part of the tumors has been reported to contain higher levels of PAI-1 antigen than more marginal parts.[41] Cell culture studies have shown that cells are able to secrete u-PA at the basolateral side, whereas PAI activity was found at apical and basolateral sides of the cells.[42] The exact distribution of u-PA and PAI-1 in liver metastases of colorectal cancer will have to be determined by immunohistochemical evaluation of tissue sections. An important role in the activation and inactivation of u-PA is probably played also by its receptor.[38,40] Preliminary studies indicated that the primary colorectal carcinomas and liver metastases contain comparable high levels of u-PA receptor.[43]

During metastasis, tumor cells must survive transport in the circulation and adhere to small blood vessels or capillaries, and may invade the vessel wall and extravasate to the organ parenchyma. Fibrin deposition around the circulating cells and the formation of small thrombi,

Table 2. Concentration of Pro–u-PA and Active u-PA in Homogenates of Primary Colorectal Carcinomas and Liver Metastases According to the Bioimmunoassay

Tissue	n	Total u-PA (*ng/mg protein*)	Pro–u-PA, (*ng/mg protein*)	Active u-PA (*ng/mg protein*)
Carcinoma colon	12	6.2 ± 1.0	4.1 ± 0.6	2.2 ± 0.9
Metastasis liver	14	2.5 ± 0.9	2.3 ± 0.7	0.3 ± 0.2

NOTE. This chromogenic assay detects u-PA by measuring the conversion of substrate by u-PA that was immunoimmobilized on microtiter plates. Total u-PA, active u-PA, and pro–u-PA activity were computed from parallel incubations with or without preincubation with plasmin. Inactivatable u-PA (complexes) is not detected by this assay. Results are given as mean ± SEM.

Table 3. Antigen Levels of u-PA, PAI-1, and PAI-2 in Homogenates of Normal Liver and Liver Disorders

Tissue	n	u-PA (*ng/mg protein*)	PAI-1 (*ng/mg protein*)	PAI-2 (*ng/mg protein*)
Normal liver, miscellaneous	11	0.4 ± 0.1	1.0 ± 0.2[a]	0.4 ± 0.2[a]
Focal nodular hyperplasia liver	3	1.8 ± 1.0	0.3 ± 0.2	1.2 ± 0.6
Liver cell carcinoma	3	1.7 ± 0.9	20.0 ± 13.3	0.8 ± 0.1

NOTE. u-PA, PAI-1, and PAI-2 antigen levels were determined with specific ELISAs. Results are given as mean ± SEM.
[a]n = 10.

consisting of tumor cells, platelets, and fibrin, may prevent their recognition and lysis by natural killer cells, which are in general responsible for the destruction of those malignant cells.[44] Apart from protection, the formation of these microthrombi could promote the successful lodgment of circulating tumor cells in small blood vessels of target organs.[45] Nevertheless, <1% of tumor cells in the circulation eventually produce metastasis.[46] Plasminogen activators could effectively participate in the process of invasion and metastasis, but too much proteolytic activity at the wrong moment could be disastrous for the survival of the metastasizing cells in a microthrombus. The balance between activators and inhibitors is essential and could therefore account for the difference in antigen levels of plasminogen activators and especially activator inhibitors between primary and metastatic tumors as shown in this study. This difference in expression in liver metastases compared with their originating colorectal carcinomas is probably the consequence of selection, external regulation, or both. The low expression of plasminogen activator activity in metastases could be a reflection of the properties of the cell(s) that gave rise to the metastatic focus[44] and was apparently an advantage for, respectively, surviving in the circulation and consecutive metastasizing, thereby supporting the microthrombus theory.[12,13] Local exposure of tumor cells to growth factors or other molecules excreted by the invaded organ are believed to influence the differentiation of the tumor cells and the expression of plasminogen activators and activator inhibitors.[47–50] Metastases vary with respect to size, growth pattern, and vascularity, depending on the primary source of the tumor. Metastases derived from colon carcinomas are usually of the expanding and massive type and frequently have central liquefactive necrosis. Besides, in most metastatic colon carcinomas, a thin collagenous pseudocapsule is often situated between the tumor margin and the compressed liver.[51] The scarcity of proteolytic activity in spite of the presence of large quantities of u-PA in metastases of colorectal carcinomas could account for the difference in tumor outgrowth between expanding metastases and more infiltrative growing primary colorectal carcinomas. Although the u-PA present in liver metastases might have a functional role in the tumor (e.g., growth-stimulating or angiogenesis-promoting),[52] it is unlikely on account of this study that u-PA in liver metastases contributes to any invasive or infiltrative process as assumed for the primary colorectal carcinomas. On the other hand, the large concentration of PAI-1 in liver metastases of colorectal carcinomas in relation to the total amount of plasminogen activator present in this tissue gives reasons to suspect a complementary alternative function for this inhibitor besides complex formation, e.g., growth promotion as shown in regenerating liver.[53,54] The relatively low levels of plasminogen activators and inhibitors that we found in other liver disorders, such as focal nodular hyperplasia and liver cell carcinomas, show that apparently the system of plasminogen activation is not uniform in tumorigenic processes of the liver.

Cell surface plasminogen activation is involved in invasion and metastasis of colorectal carcinomas. Regulation of this process is provided at multiple levels. Although the expression and activation of pro–u-PA seem to be key events, it is clear that dominant regulating roles in the process are played by the inhibitors and probably also by the u-PA receptor. It is expected that understanding of the regulation of plasminogen activation will eventually contribute to the treatment and prevention of tumor dissemination and metastasis.

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